

Atopic March from Atopic Dermatitis to Asthma-Like Lesions in NC/Nga Mice Is Accelerated or Aggravated by Neutralization of Stratum Corneum but Partially Inhibited by Acidification

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Prolonged and/or repeated damage to the skin barrier followed by atopic dermatitis (AD) is an initial step in atopic march that ultimately progresses to respiratory allergy. Maintaining normal stratum corneum (SC) acidity has been suggested as a therapeutic or preventive strategy for barrier impairment caused by skin inflammation. We determined whether a representative AD murine model, NC/Nga mice, develops airway inflammation after repeated epicutaneous application followed by inhalation of house dust mite (HDM), implying atopic march, and whether prolongation of non-proper SC acidity accelerates respiratory allergy. HDM was applied to the skin of NC/Nga mice, accompanied by the application of neutral cream (pH 7.4) or acidic cream (pH 2.8) for 6 weeks. Intranasal inhalation of HDM was administered daily during the last 3 days. Repeated epicutaneous applications followed by inhalation of HDM in NC/Nga mice induced an atopic march-like progression from AD lesions to respiratory allergy. Concurrent neutral cream treatment accelerated or aggravated the allergic inflammation in the skin and respiratory system, whereas an acidic cream partially alleviated these symptoms. Collectively, we developed an atopic march in NC/Nga mice by HDM application, and found that prevention of a neutral environment in the SC may be an interventional method to inhibit the march.

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INTRODUCTION

Atopic dermatitis (AD), which is one of the most common chronic inflammatory dermatosis, is characterized by an impaired epidermal barrier, dry skin, pruritus, eczematous lesions, abnormal immune response, and IgE-mediated allergy to various exogenous antigens (Lee and Lee, 2014). The clinical symptoms of AD generally precede the development of asthma and allergic rhinitis. This finding suggests that AD could be a doorway to the successive events of allergic diseases, the so called “atopic march” (Spergel and Paller, 2003; Gordon, 2011). Researchers have suggested that the occurrence of asthma in AD patients could result from enhanced sensitization of allergens through a defective skin barrier, enabling allergens to penetrate the skin easily, and subsequently to come in

contact with to contact with epidermal Langerhans cells (LCs), antigen presenting cells (Callard and Harper, 2007; Zheng *et al.*, 2011). After capturing and processing the penetrated exogenous allergens, LCs move to regional lymph nodes and present the allergens to naïve T cells, which leads to systemic allergy by encouraging Th2 inflammation (Allakhverdi *et al.*, 2007). Besides, thymic stromal lymphopoietin (TSLP) secreted by the keratinocytes of disrupted epidermis activates polyclonal T cells. These T cells, in turn, induce excessive Th2 inflammatory responses, which could aggravate the AD and trigger the systemic Th2 responses including respiratory allergies (Laouini *et al.*, 2003).

The acidity (pH) of the stratum corneum (SC) affects the epidermal key functions including permeability barrier homeostasis, SC integrity and cohesion, initiation of inflammation, processing of the secreted lamellar body (LB) lipids, and antimicrobial defenses (Hachem *et al.*, 2010; Elias, 2015). The skin surface of AD lesions exhibits an increased pH, which causes bacterial growth and results in allergic inflammation and worsening of AD. In a hapten-induced animal model of AD, simple acidification of SC significantly blocks the progression of barrier impairment and the downstream immune abnormalities (Hatano *et al.*, 2009). These studies give us a clue to possible novel modalities that might prevent progression of the atopic march (Lee *et al.*, 2014).

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Abbreviations: AD, atopic dermatitis; Dp, Dermatofagoides pteronissunus; FLG, filaggrin gene; HDM, house dust mite; LC, Langerhans cell; PAR-2, protease activated receptor-2; SC, stratum corneum; SLS, sodium lauryl sulfate; TEWL, transepidermal water loss; TSLP, thymic stromal lymphopoietin

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Acidic microdomain was observed within the extracellular matrix of the SC by two-photon fluorescence lifetime imaging. The mean pH of the SC increased with depth owing to an increase in the ratio of neutral to acidic regions within the SC. Fluorescence lifetime imaging definitely showed that the existence of aqueous acidic pockets within the lipid-rich extracellular matrix leads to the acid mantle of the SC (Hanson *et al.*, 2002). It also revealed that SC acidification occurs through the progressive accumulation of acidic microdomains (Behne *et al.*, 2002). These results give us a clue that surface pH directly reflects changes in the extracellular matrix of the SC as an acidic pH microdomain in the SC. The abnormal acidification demonstrated by fluorescence lifetime imaging is related to decreased NHE1 expression in the epidermis of moderately aged mice. Diminished Na^+/H^+ antiporter levels cause the elevation of SC pH, which contributes to suboptimal activation of β -glucocerebrosidase, leading to defective maturation of lipid processing and lamellar membranes in turn. These abnormalities were normalized by exogenous acidification of the SC (Choi *et al.*, 2007).

The NC/Nga mouse has a mutation on chromosome 9, which is related to the increased IgE production and a Th2 dominant inflammation. The skin lesions arise spontaneously or secondarily to exposure of various aeroallergens, which are similar to AD in humans. The mice also present abnormalities of skin barrier functions, such as increased basal transepidermal water loss (TEWL) and abnormal SC hydration under conventional conditions, and diminished ceramide metabolism (Aioi *et al.*, 2001). In addition, exposure to a standard environment alone is not enough to reveal AD-like symptoms in these mice; however, epicutaneous stimulation with certain environmental allergens (e.g., house dust mite allergens) provokes AD-like symptoms (Kim *et al.*, 2014).

Based on previous reports, we first hypothesized that NC/Nga mice, as a murine model of inherited AD, would establish respiratory inflammation after epicutaneous treatment followed by subsequent inhalation of house dust mite (HDM), similar to the model of the atopic march. Second, we hypothesized that the atopic march could be interrupted by maintaining SC acidity or accelerated by neutralization of the SC.

RESULTS

Repeated epicutaneous application of HDM extract provokes remarkable AD-like skin lesions and barrier dysfunction in NC/Nga mice, a representative murine model of inherited AD, which are aggravated by a neutral cream

Repeated epicutaneous application of a HDM, *Dermatofagoides pteronyssinus* (Dp) antigen provoked remarkable AD-like skin lesions with a high eczema score in the NC/Nga mice but not in the control mice (BALB/c mice). BALB/c mice did not exhibit eczema lesions despite epicutaneous treatment of SLS and Dp antigen. Treatment with SLS alone to NC/Nga mice also induced eczema lesions. Moreover, application of a neutral cream with Dp extract (NC/Nga SLS+Dp+pH 7.4 cream) resulted in more prominent eczema lesions compared with other NC/Nga treatment groups. Time course of skin

surface pH after the application of creams on the skin of NC/Nga mice revealed that skin surface pH is maintained by acidic or neutral cream for ~6 hours after application of the creams (Figure 1a). Application of an acidic cream (NC/Nga SLS+Dp+pH 2.8) alleviated eczema lesions in Dp-treated NC/Nga mice, but this effect was not significant (Figure 1b). Post-treatment basal TEWL was increased and SC hydration was significantly decreased in the group treated with neutral cream (NC/Nga SLS+Dp+pH 7.4 group; Figure 1c and d). However, there was no significant difference in skin surface pH among all groups, measured 24 hours after the final application of Dp, that is, 12 hours after the final application of cream (Figure 1e).

Systemic and cutaneous allergic inflammation and abnormal epidermal differentiation induced in NC/Nga mice are aggravated by neutral cream but partially improved by acidic cream

After treatment, serum total IgE levels significantly increased in all of the NC/Nga mice compared with control BALB/c mice. Among NC/Nga mice, mice that were treated with neutral cream showed the most prominent increase in serum IgE. In contrast, acidic cream did not decrease serum IgE in NC/Nga mice treated with SLS and Dp antigen (Figure 1f). Post-treatment serum TSLP was significantly increased in the NC/Nga SLS+Dp group. Mice treated with neutral cream (NC/Nga SLS+Dp+pH 7.4) showed an increase in serum TSLP similar to that of the NC/Nga SLS+Dp group. Application of acidic cream significantly decreased serum TSLP in the NC/Nga SLS+Dp group (Figure 1g).

In addition, the number of tissue eosinophils was noticeably increased in the skin of NC/Nga SLS+Dp+pH 7.4 group (Figure 2a). Semiquantitative analysis of the expression of epidermal differentiation markers (filaggrin, involucrin, and loricrin) by immunohistochemical staining showed that their expression was significantly decreased in the NC/Nga SLS+Dp group compared with the control BALB/c SLS+Dp group (Figure 2b). Application of neutral cream to the NC/Nga SLS+Dp group significantly decreased expression of these markers, whereas acidic cream significantly increased their expression. Semiquantitative analysis demonstrated that epidermal expression of protease activated receptor—2 (PAR-2) and TSLP significantly increased in the NC/Nga SLS+Dp group compared with the control BALB/c SLS+Dp group (Figure 2b). Application of neutral cream to the NC/Nga SLS+Dp group significantly increased expression of PAR-2 and TSLP, whereas acidic cream significantly decreased their expression.

Repeated epicutaneous application followed by nasal inhalation of HDM extract produces asthma-like airway inflammation and AD-like skin lesions in NC/Nga mice, implying atopic march; these symptoms are aggravated by neutral cream and partially improved by acidic cream

Because airway eosinophilia is a characteristic manifestation of asthma (Tian *et al.*, 2011), we performed bronchoalveolar lavage after 3 consecutive days of nasal inhalation of Dp. After treatment, the total cell count of the bronchoalveolar

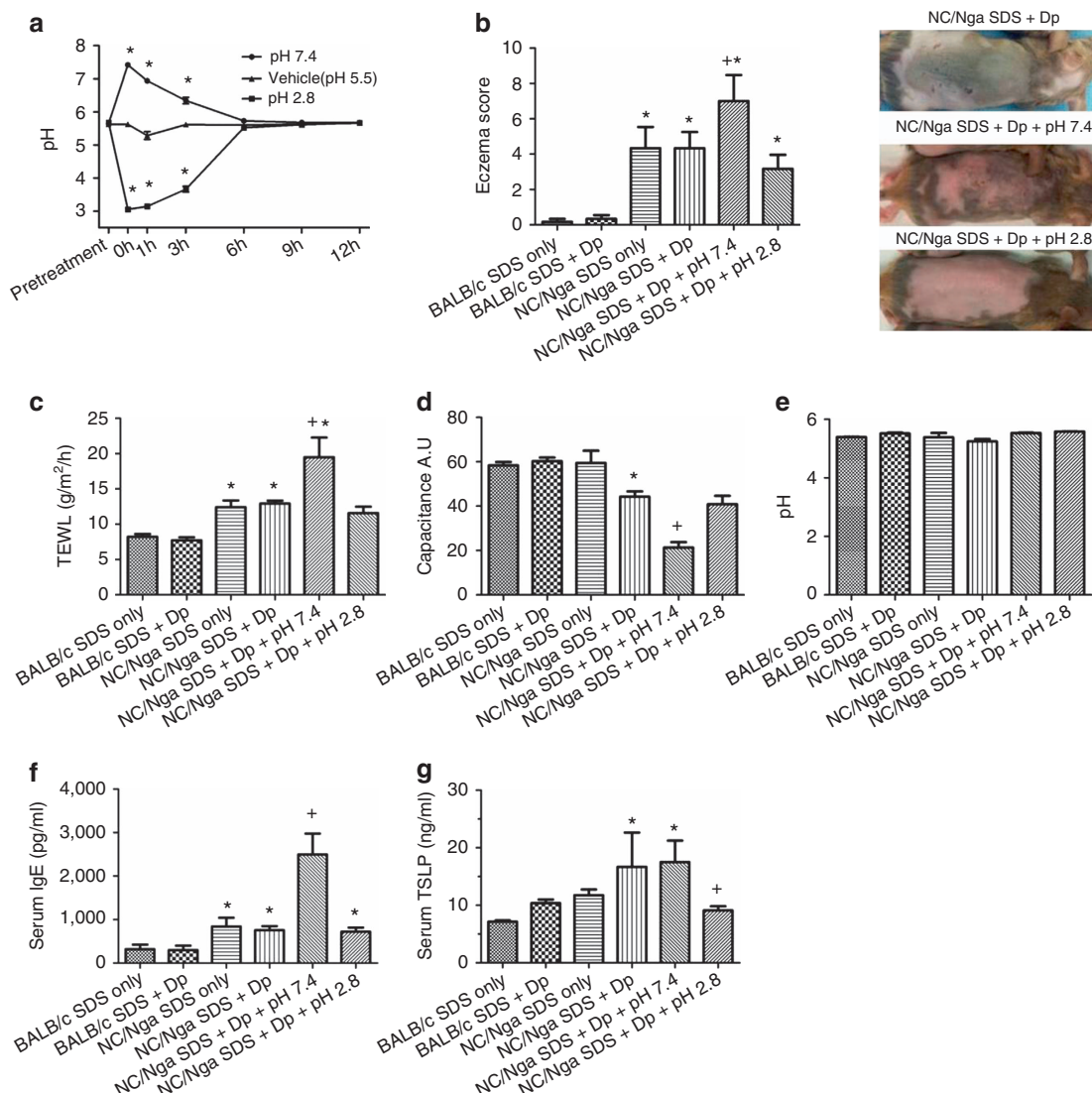


Figure 1. Repeated epicutaneous application of HDM (Dp) extract followed by nasal inhalation provokes remarkable AD-like lesions and barrier disruption in NC/Nga mice. Symptoms were aggravated by neutral cream, but neither aggravated nor improved by acidic cream. Systemic allergic inflammation was aggravated by neutral cream but partially improved by acidic cream. (a) Time course of skin surface pH after application of acidic or neutral cream ($n=4$, each group). (b) Eczema scores and gross appearance, (c) basal TEWL, (d) SC hydration, (e) skin surface pH, (f) serum IgE, and (g) TSLP after Dp treatment were compared among treatment groups. ANOVA showed $P<0.05$ in all measurements except (e) surface pH. Results are mean $\pm$ SEM. * $P<0.05$, compared with control (BALB/c SLS+Dp); + $P<0.05$, compared with NC/Nga SLS+Dp. ($n=6$, each group). ANOVA, analysis of variance; SC, stratum corneum; TEWL, transepidermal water loss.

lavage was significantly increased in the NC/Nga SLS+Dp group; this increase was aggravated by treatment with neutral cream (NC/Nga SLS+Dp+pH 7.4 group) and decreased by application of the acidic cream with statistical significance (Figure 3a). The number of eosinophils, macrophages, and neutrophils were significantly increased in the NC/Nga SLS+Dp group, similar to the results of total cell count (Figure 3b). Quantitation of mRNA and immunohistochemical staining revealed increased expression of PAR-2 and TSLP in the lung tissue of the NC/Nga SLS+Dp group, which was further aggravated by application of neutral cream application and decreased by acidic cream with statistical significance (Figure 3c, d and e).

DISCUSSION

As Strid *et al.* suggested the idea of a skin barrier defect initiating allergic sensitization and showed its mechanism in mice (Strid *et al.*, 2006), the theoretical model described by Kubo *et al.* (Kubo *et al.*, 2012) has been considered applicable to the atopic march pathogenesis. Break down of the epidermal barrier activates the intrinsic proteases and permits the penetration of protease-active allergens, which could activate Toll-like receptors and PAR-2 on keratinocytes (Briot *et al.*, 2009). The keratinocytes subsequently produce proinflammatory cytokines including IL-1, TNF- α , and TSLP. Along with the disruption of SC, the activated LCs extend their dendrites through the weakened tight junctions and attach to

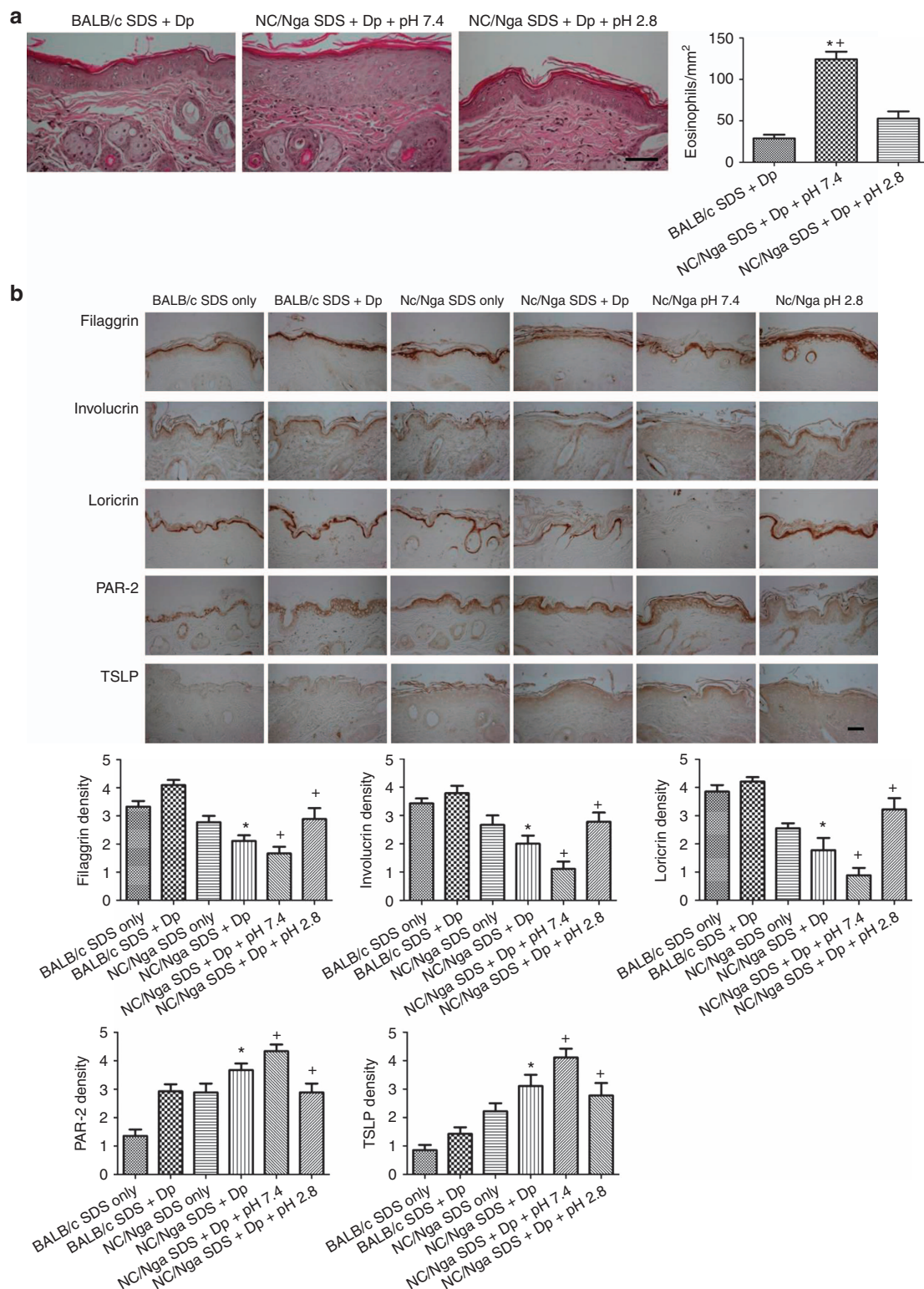


Figure 2. Cutaneous allergic inflammation and abnormal epidermal differentiation induced in NC/Nga mice are aggravated by neutral cream but partially improved by acidic cream. (a) Tissue eosinophils were counted with H&E staining, and (b) expression of epidermal differentiation markers (filaggrin, involucrin, loricrin), PAR-2, and TSLP were measured by immunohistochemical staining in skin specimens taken after repeated epicutaneous treatment and nasal inhalation of Dp. (a) ANOVA and (b) Kruskal–Wallis test showed $P < 0.05$ in all measurements. Results are mean $\pm$ SEM. * $P < 0.05$, compared with control (BALB/c SLS+Dp); + $P < 0.05$, compared with NC/Nga SLS+Dp. ($n = 6$, each group). ANOVA, analysis of variance; SLS, sodium lauryl sulfate; TSLP, thymic stromal lymphopoietin.

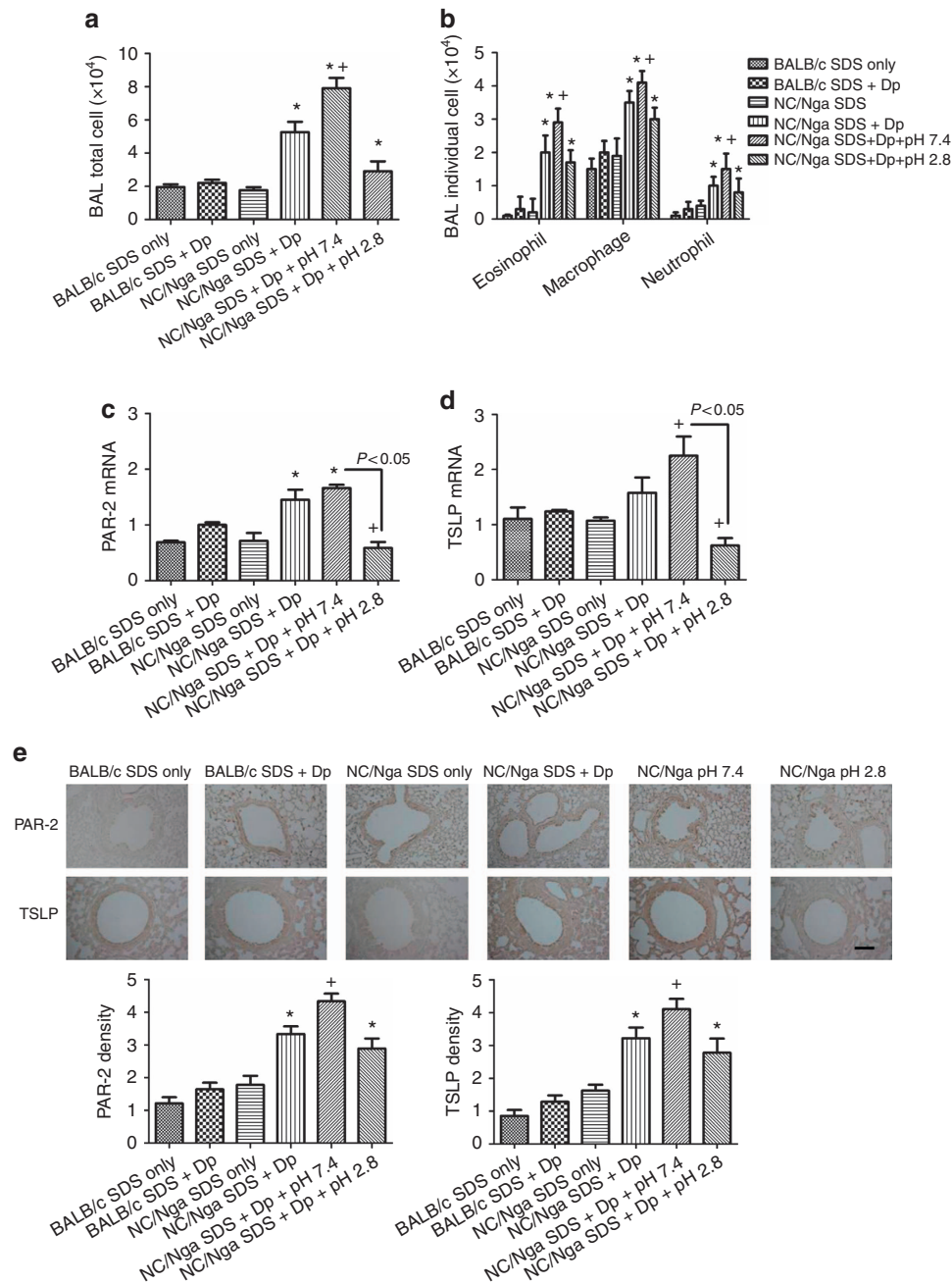


Figure 3. Repeated epicutaneous application of house dust mite (Dp) extract followed by nasal inhalation produces asthma-like inflammatory response as well as AD lesions in NC/Nga mice, implying atopic march. Symptoms are aggravated by neutral cream, but partially improved by acidic cream. BAL, real time quantitative-PCR for mRNA expression, and immunohistochemical staining of PAR-2 and TSLP were performed in the lung tissue after Dp treatment. (a) Total cells and (b) individual cell count in the BAL, and (c and d) mRNA and (e) expression of PAR-2 and TSLP in the lung tissue were measured. (a–d) ANOVA and (e) Kruskal–Wallis test showed $P < 0.05$ in all measurements. Results are mean $\pm$ SEM. * $P < 0.05$, compared with control (BALB/c SLS+Dp); + $P < 0.05$, compared with NC/Nga SLS+Dp and NC/Nga SLS+Dp+pH 2.8. ($n = 6$, each group). ANOVA, analysis of variance; BAL, bronchoalveolar lavage; SLS, sodium lauryl sulfate; TSLP, thymic stromal lymphopoietin.

the penetrating allergens (Kubo *et al.*, 2009). Such percutaneous sensitization of exogenous allergens, followed by repeated challenges from various routes, including lung, nasal cavities, and gastrointestinal tract, is thought to develop the atopic march. In our study, only epicutaneous treatment and nasal inhalation of HDM with no disruption of

permeability barrier in the control group of BALB/c mice did not demonstrate such remarkable inflammatory lesions in the skin, as well as the lung (Figures 1,2 and 3). This result suggests that the uptake of active allergens and the activation of LCs did not take place when the epidermal barrier was not disrupted, therefore there was no increase in the levels of

cytokines and TSLP. It is well known that the expression of TSLP is highly increased in lesional skin and the blood of patients with AD, and that this elevation in TSLP expression is sufficient to activate LCs during AD development (Liu, 2006; Kubo *et al.*, 2012).

There have been many attempts to develop animal models of atopic march. Although all of the results present asthma-like lung lesions as well as AD-like skin lesions, intraperitoneal injections of ovalbumin were used for sensitization rather than epicutaneous application via a disrupted barrier (Li, 2014). To develop asthma-like lung lesions, HDM antigen was treated to AD lesions presenting a broken barrier before the nasal inhalation in NC/Nga mice, a murine model of inherited AD. This provided a more natural and physiologic mechanism for allergen sensitization compared with previous sensitization methods. Recently, we reported that a murine model of acquired AD induced by repeated hapten (oxazolone) application progresses to asthma-like lesions under more natural and physiologic mechanisms (Lee *et al.*, 2014).

The NC/Nga mouse shows an abnormal skin permeability barrier, which presents increased TEWL and abnormal skin hydration. These are combined with Th2 inflammatory responses and impaired ceramide metabolism even in normal condition (Matsuda *et al.*, 1997). Our results show that the eczema score was increased in the group of NC/Nga mice treated with SLS only, which confirms that NC/Nga mice have inherited abnormalities in skin barrier functions. Their AD-like skin changes are suggested to be independent of IgE and Th2 reaction, as the skin lesions developed in STAT6-deficient NC/Nga mice with undetectable IgE serum levels (Yagi *et al.*, 2002). Although the Th2 response may not be necessary for AD development in NC/Nga mice, additional applications of SLS and HDM may cause earlier barrier disruption and allergen exposure, which could contribute to the Th2 immune response. Therefore, in our study a greater increase in eczema scores was associated with poorer barrier functions and a greater increase in serum TSLP and IgE.

In AD lesions, filaggrin expression is downregulated by Th2 cytokines such as IL-4, IL-13, IL-22, and IL-25 regardless of *FLG* gene mutations and the decrease in NMF is dependent on the severity of lesional inflammation, rather than the *FLG* mutation (Jung *et al.*, 2014; Novak, 2012). In addition, epidermal differentiation in AD lesions is incomplete (Elias, 2010). In this atopic march-like model using NC/Nga mice, the expression of filaggrin, involucrin and loricrin decreased after multiple epicutaneous applications of HDM antigens. Repeated application of HDM antigen to the NC/Nga mouse model aggravated AD skin lesions and then severely decreased filaggrin expression, which could disrupt the skin barrier and eventually permit skin permeation of exogenous antigens including HDM (Figure 2b). This process may also result in the asthma-like lesions.

PAR-2 and TSLP expression has been demonstrated in various tissues in addition to skin and lung, which are also involved in the occurrence of AD and respiratory allergy (Ebeling *et al.*, 2005; Gu and Lee, 2012; Lee *et al.*, 2014). TSLP expression is induced in airway epithelial cells following

exposure to allergen-derived proteases, and PAR-2 has a role in this process (Kouzaki *et al.*, 2009). In our study, repeated HDM application to damaged skin increase the levels of TSLP, IgE, or PAR-2 in the systemic circulation or lung tissue (Figure 3). Based on our results, we presume that HDM-induced asthma could occur more easily when the skin barrier is more severely impaired and AD lesions are more severe. These conditions provoke an increase in systemic TSLP and IgE as well as eosinophils, whereas HDM application alone does not easily induce asthma. Systemic TSLP levels may affect the PAR-2 and TSLP in distant epithelial organs such as the lung, which might be readily affected by the HDM allergen.

There are controversies as to whether filaggrin deficiency per se increases the SC pH or not (Ohman and Vahlquist, 1998; Fluhr *et al.*, 2010; Vávrová *et al.*, 2014). However, decrease in filaggrin degradation products in AD lesions might contribute to an increase in the SC pH high enough to induce serine proteases activation in the SC which optimally acts under neutral to alkaline pH (Brattsand *et al.*, 2005). An elevated SC pH also increases the activity of serine proteases such as kallikrein 5 and KLK7 (stratum corneum chymotryptic enzyme), resulting in overdegradation of corneodesmosomes and decrease of SC integrity and cohesion (Lee *et al.*, 2010). On the other hand, the elevation of SC pH decreases the activity of lipid-processing enzymes, which are indispensable for the production of free fatty acids and ceramide in the SC, and thus impairs lipid processing and eventually disrupts the permeability barrier of epidermis (Hatano *et al.*, 2009; Hachem *et al.*, 2010).

Several reports present some indirect evidence that acidification of SC prevents the penetration of the SC by external allergens including HDM. This evidence was demonstrated with ultrastructural changes of SC and stratum granulosum shown by fine electron microscope pictures (Hachem *et al.*, 2003, 2005, 2010; Hatano *et al.*, 2009). Therefore, we believe that allergens from Dp extract with high molecular weight over 18,000 dalton (Lind, 1985) only limitedly penetrate the skin barrier, which is partially improved by SC acidification in NC/Nga mice. Hatano *et al.* reported that an acidic environment can inhibit the formation of AD-like skin lesions in the hapten-induced AD model (Hatano *et al.*, 2009). We previously reported that acidification of SC inhibits the development of both asthma-like lesions and AD-like lesions by HDM in the murine model (Lee *et al.*, 2014). In the present study using NC/Nga mice, we observed an aggravating or harmful effect of neutral cream and a partial preventive effect of acidic cream on the atopic march. A previous study already confirmed that acidic treatment (pH 2.0 with 2M HCl) to HDM (Dp) preserved the allergenicity and antigenicity of Dp. No significant differences were observed in the antigenic profile of native and acid treated extracts (Fernández-Caldas *et al.*, 2008). The difference in the degree of response might reflect differences in skin structure between these mouse models. Compared with hairless mice, NC/Nga mice with abundant terminal hair have a thinner SC and weaker skin barrier function. Also, the transient receptor potential vanilloid type 1 (TRPV1) channel,

which is activated by diverse noxious stimuli including capsaicin, low pH or heat, is deeply associated with permeability barrier function. Blockade of its activation accelerates barrier recovery and alleviates AD-like symptoms in NC/Nga mice and Ox-AD mice (Yun *et al.*, 2011). Therefore, we assume that the acidic cream might more easily activate TRPV1 in the epidermis of NC/Nga mice with relatively thinner SC compared with hairless mice. Another considerable factor is that AD-like skin changes in NC/Nga mice are IgE/Th2-independent, which is different with AD lesions of hairless mice and humans (Yagi *et al.*, 2002). This immunologic difference might be the cause of the partial or minimal pH-dependent preventative effects in the NC/Nga mice, and might also explain the lack of statistical difference in surface skin pH among the groups. In addition, skin surface pH was maintained approximately for 6 hours after application of the creams, but the creams were applied every 12 hours (Figure 1a). Further study is needed to evaluate whether the acidic cream is more effective if applied every 6 hours.

In summary, we demonstrate that a murine model of atopic march can be developed by application of HDM in NC/Nga mice. Maintenance of a neutral skin environment aggravates barrier function and AD lesions which in turn, eventually, accelerates the atopic march. Therefore, prevention of a neutral environment on the SC may be an intervention to inhibit the progression of AD to respiratory allergies, an atopic march.

MATERIALS AND METHODS

Animals and materials

All animal procedures were approved by the Yonsei University Wonju Campus Institutional Animal Care and Use Committee (IACUC). Twenty-four female NC/Nga mice (5-weeks-old) were purchased from SLC Japan (Shizuoka, Japan). Mice were kept under conditions of controlled humidity (40%) and temperature (22 ± 2 °C). Extracted antigen of *D. pteronyssinus* (Dp, a HDM, was purchased from the Department of Environmental Medical Biology, Yonsei University (Seoul, Korea). All laboratory measurements were performed under blinded conditions. An acidic cream (pH 2.8) was made by the addition of citric acid (Sigma-Aldrich; St Louis, MO) to

vehicle cream. A neutral cream (pH 7.4) was made by neutralization of the acidic cream with sodium hydroxide (NaOH). The vehicle was plain Cetaphil cream with a pH of 5.5 (Galderma, Lausanne, Switzerland), as previously described (Jung *et al.*, 2011).

Development of an atopic march model in NC/Nga mice by epicutaneous application and subsequent nasal inhalation of Dp, application of acidic or neutral cream, and measurement of skin barrier function including surface pH

Five-week-old NC/Nga mice and BALB/c mice (as a control) were used in this study. Once a week the mice were anesthetized and the hair on their dorsal skin was shaved. Dp antigen (Ag) was mixed with petrolatum to make a 0.5% ointment. Four percent SLS and 0.5% Dp Ag were applied to the dorsal skin of the NC/Nga mice (NC/Nga+Dp) and the control mice (BALB/c+Dp) twice a week for 6 weeks (a total of 12 applications). During the last 3 days of the experiment, 100 µg of Dp Ag was given daily by intranasal inhalation (Lee *et al.*, 2014). Although Dp Ag was being applied epicutaneously, 200 mg of acidic cream (pH 2.8) was applied twice a day on the same skin sites for 45 days (acidic cream group). For the non-treated control groups (BALB/c), no cream was applied. For the neutral cream group, neutralized cream (pH 7.4) was applied twice a day (Figure 4). After a total of 46 days of epicutaneous and intranasal application with Dp Ag, the gross appearance and barrier functions of the skin, including basal TEWL and SC hydration, skin surface pH, and the degree of allergic inflammation of the skin, blood, and respiratory system were evaluated in all mice. Surface pH was measured with pH meter (WTW, Weilheim, Germany). The basal TEWL was measured with the Tewameter TM210 (Courage and Khazaka, Cologne, Germany), and SC hydration was assessed as capacitance with the Corneometer CM820 (Courage and Khazaka) 24 hours after the final application of Dp, that is 12 hours after the final application of cream, as described previously (Choi *et al.*, 2005; Lee *et al.*, 2014). Twenty-four hours after the last treatment of Dp, specimens of skin, blood, and lung were collected to evaluate the degree of allergic inflammation as measured by mRNA and protein expression. In addition, to evaluate the maintenance time of lowered or elevated pH by application of acidic or neutral cream, skin surface pH was measured at pretreatment and 0, 1, 3, 6, 9, and 12 hours right after the application of acidic (pH 2.8) or neutral (pH 7.4) creams as well as vehicle (pH 5.5) cream. (each group, $n = 4$).

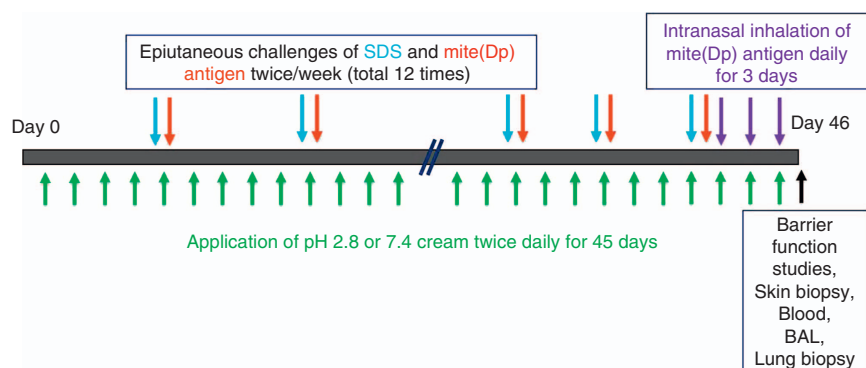


Figure 4. Establishment of a model of atopic march in NC/Nga mice by epicutaneous application and subsequent nasal inhalation of house dust mite, and the intervention by topical acidic or neutral cream.

Evaluation of macroscopic AD-like skin lesions

Scoring of dermatitis was assessed macroscopically in a blinded fashion using the following scoring procedure. The development of (i) erythema, (ii) edema, (iii) excoriation, (iv) scale, and (v) lichenification was each scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The total symptom severity score was defined as the sum of the individual scores.

Tissue preparation, immunohistochemistry, and immunofluorescence

Immunohistochemical staining was performed to assess epidermal differentiation and inflammation. Briefly, 5- μ m thick paraffin sections were incubated with primary antibodies against epidermal differentiation marker proteins such as filaggrin, involucrin, and loricrin, (PAR-2, and TSLP (all from Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After three cycles of washing, the sections were incubated with the appropriate secondary antibody for 30 minutes. Staining was detected with the ABC-peroxidase kit (Vector Lab, Burlingame, CA) and counterstaining with hematoxylin was performed. To quantify the density of immunohistochemical staining, three pictures from each section were taken at $\times 100$ magnification and labeled. An investigator without any information on the specimen ranked the pictures in order of the intensity of immunostaining (the highest intensity received the first rank). Representative images of each group are provided.

ELISA for serum IgE and TSLP

Serum levels of IgE and TSLP were determined using ELISA kits for mouse IgE (Koma Biotech, Seoul, Korea) and mouse TSLP (R&D systems, Minneapolis, MN). To detect serum IgE levels, whole blood samples were centrifuged at 5,000 r.p.m. for 5 minutes at room temperature. The sample serum and standard solutions were added to plates that were pre-coated with specific antibodies for IgE and TSLP, and incubated for 1 hour at room temperature. Horseradish peroxidase-conjugated mouse secondary detection antibodies were added to each well and incubated for 1 hour at room temperature. TMB substrate was added to each well and incubated for 20 minutes for color development. A sulfuric acid solution was added to each well to stop the reaction and the enzyme-substrate reaction was measured using a microtiter plate reader (BioTek Instruments, Inc., Winooski, VT) at a wavelength of 450 ± 2 nm. After triplicate measurements, the concentrations of IgE and TSLP in the samples were determined by comparison with a standard curve.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously described (Lee et al., 2014). After each mouse was anesthetized, the trachea was cannulated and the lung was lavaged with 1 ml of Hank's balanced salt solution via the intubation tube. Bronchoalveolar lavage fluid was obtained by two rounds of aspiration. Total numbers of cells were counted and cell smear preparations were made. Cells were stained with Wright-Giemsa stain and eosinophils, lymphocytes, macrophages, and neutrophils were counted and expressed as a percentage of 400 total cells. The counts of eosinophils, lymphocytes, macrophages, and neutrophils in the bronchoalveolar lavage fluid were determined by multiplying their percentages in total cells.

Total RNA preparation and cDNA synthesis with blood and lung tissue, and Quantitative PCR analysis of gene expression

Total RNA preparation and cDNA synthesis were performed as previously described (Jung et al., 2011; Lee et al., 2012). Expression of specific mRNAs was quantified using a Rotor-Gene 3000 (Corbett Life Science, Brisbane, Qld, Australia), as in our previous reports (Hong et al., 2010; Jung et al., 2011; Lee et al., 2012). Briefly, 10- μ l PCR reactions were set up containing Quantitect SYBR green PCR kit Master mix (Qiagen, Hilden, Germany) in a 2 $\times$ solution, 8 mM manganese chloride, 200 μ M deoxynucleotide triphosphates, 1.25 units Hotstart Taq polymerase, and 0.5 pM μ l⁻¹ each of probes and primers. Approximately 60 ng of cDNA was used per reaction. Primers for PAR-2 and TSLP were designed based on GeneBank sequences using Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>, Massachusetts Institute of Technology, Cambridge, MA). All reactions included GAPDH as a housekeeping gene. Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines and were used to determine Δ Ct values (Ct of target gene – Ct of housekeeping gene) as raw data for gene expression. Changes in gene expression were determined by subtracting Δ Ct values for samples and acidic cream-treated samples from their respective control samples. The resulting $\Delta\Delta$ Ct values were used to calculate fold change in gene expression as $2^{-\Delta\Delta$ Ct}. All reactions were performed in triplicate, and the results are expressed as the mean of values from three separate experiments. Samples were amplified under the following conditions: 95 °C for 15 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

Statistical analysis

All data were expressed as mean $\pm$ SEM. Analysis of variance or Kruskal–Wallis test were performed when three or more groups were compared or to compare the density of immunohistochemical staining. For individual comparison, Student's unpaired *t*-test was used in case of normal distribution, and Mann–Whitney test was used in case of non-normal distribution or ranking variables. **P* < 0.05 was regarded as statistically significant. Statistical analysis was conducted using the SPSS software 10.1 (SPSS, Chicago, IL).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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